Recombinant Expression and Use in Serology of a Specific Fragment from the *Cowdria ruminantium* MAP1 Protein[a]

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INTRODUCTION

Cowdria ruminantium is the causative agent of cowdriosis (heartwater), a rickettsial disease of domestic and wild ruminants. The disease is transmitted by ticks of the genus *Amblyomma*.[1] Cowdriosis is endemic in sub-Saharan Africa, and there it is a major obstacle for upgrading local breeds of livestock with more productive susceptible exotic breeds.[1] The disease is also present on at least three Caribbean islands,[2-4] and poses a threat to livestock production on the North and South American mainland.[5]

Several serological tests have been developed for detection of antibodies to *C. ruminantium*. These include immunofluorescence assays,[6-8] an indirect ELISA based on *in vitro* cultured *C. ruminantium* organisms,[9] and two assays based on the immunodominant major antigenic protein of *C. ruminantium* (MAP1,[10] formerly called Cr32[11]).

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These assays were either a competitive ELISA[12] or an immunoblotting assay.[13,14] However, cross-reactivity between *C. ruminantium* antigens and antibodies to several *Ehrlichia* species has been observed in all these tests.[13-17] This cross-reactivity is problematic, as shown by discrepancies between the distribution of *Amblyomma variegatum* ticks and the presence of antibodies to *C. ruminantium*.[13,18-20] As a consequence, a serological test with improved specificity was urgently required for a better understanding of the epidemiology of heartwater.

The recent cloning and expression of the MAP1 gene of *C. ruminantium*[21] allowed identification of immunogenic regions on the MAP1 protein, and evaluation of their use in the construction of a specific serological assay for cowdriosis. This study describes the development and validation of such an ELISA based on recombinant MAP1 antigens.

MATERIALS AND METHODS

Rickettsial and Bacterial Strains

C. ruminantium isolates used in this study were from Senegal (Senegal),[22] South Africa (Welgevonden),[23] and Guadeloupe (Gardel).[24] *C. ruminantium* was cultivated in bovine umbilical endothelial cells as described previously.[25] The *E. ovina* isolate has been described previously.[14] *Escherichia coli* strain M15[pREP4] was used as host strain for plasmid pQE9 (Qiagen Inc., Chatsworh, CA) and grown in LB-broth supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin.

Molecular Techniques

All DNA manipulations, cloning experiments, and SDS-polyacrylamide gel electrophoresis were carried out according to standard methods.[26]

Antisera

Antibodies to *C. ruminantium* or *E. ovina* were raised in experimentally infected sheep by an infection and treatment method.[27] Infection with *C. ruminantium* or *E. ovina* was done by intravenous injection of virulent blood. Field serum panels used were obtained from Zimbabwe and several Caribbean islands. The Zimbabwean panel consisted of 55 immunoblot-negative and 111 immunoblot-positive sheep sera from heartwater-free areas on the highveld of Zimbabwe.[13] This panel was supplemented with 10 serum samples from sheep experimentally infected with *C. ruminantium* (Welgevonden isolate). The panel from the Caribbean islands consisted of 81 *C. ruminantium* indirect ELISA positive sheep sera and 7 indirect ELISA negative sheep sera.[9,20] This panel was supplemented with one serum sample from a sheep experimentally infected with *C. ruminantium* (Gardel isolate).

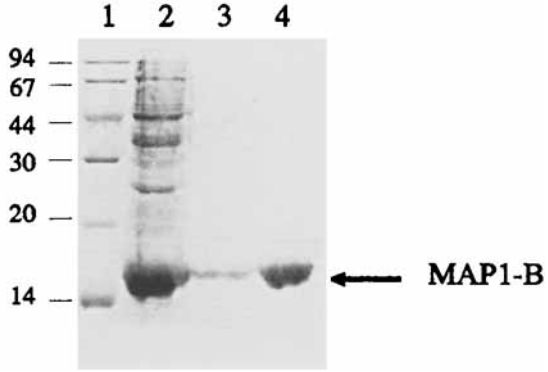

FIGURE 1. Purification of recombinant MAP1-B, demonstrated by using SDS-polyacrylamide gel electrophoresis. Lane 1, molecular weight markers; lane 2, *E. coli* cells expressing MAP1-B; lane 3, MAP1-B eluted at pH 8.0; lane 4, MAP1-B eluted at pH 6.3.

Serology

Identification and expression of MAP1-A and MAP1-B immunogenic regions are described elsewhere.[28] Briefly, PCR fragments encoding either amino acids 1-152 or 47-152 of the mature MAP1 protein[21] fused with 6 consecutive histidine residues were expressed in *E. coli* using the QiaExpress vectors (Qiagen Inc., Chatsworth, CA). Recombinant MAP1-A and MAP1-B fusion proteins were purified using Ni^{2+}-NTA agarose under denaturing conditions as described by the manufacturer (Qiagen Inc., Chatsworth, CA). Immunoblotting[14] and MAP1-B ELISAs[28] were performed as described elsewhere. Reactivity of antisera in the MAP1-B ELISA was expressed as a percentage of a high-titered positive control serum (% C++, see FIGS. 3, 4, and 5) as recommended elsewhere.[29]

RESULTS

Specificity of Immunogenic Regions on the C. ruminantium *MAP1 Protein*

Immunogenic regions on the MAP1 protein were identified after recombinant expression of overlapping fragments of the MAP1 protein in *E. coli* and subsequent immunoblotting with antisera to *C. ruminantium* and *E. ovina*.[28] Two immunogenic regions were identified, designated MAP1-A and MAP1-B.[28] They were expressed with 6 consecutive histidine residues (6*His) added at the N-terminus to obtain them in a suitable form for a serological assay. Using this system, recombinant MAP1 proteins could be easily purified from contaminating *E. coli* proteins due to binding of the 6*His tag to nickel. FIGURE 1 shows a typical purification of MAP1-B, with no contaminating proteins visible after elution from Ni^{2+}-NTA agarose (FIG. 1). FIGURE

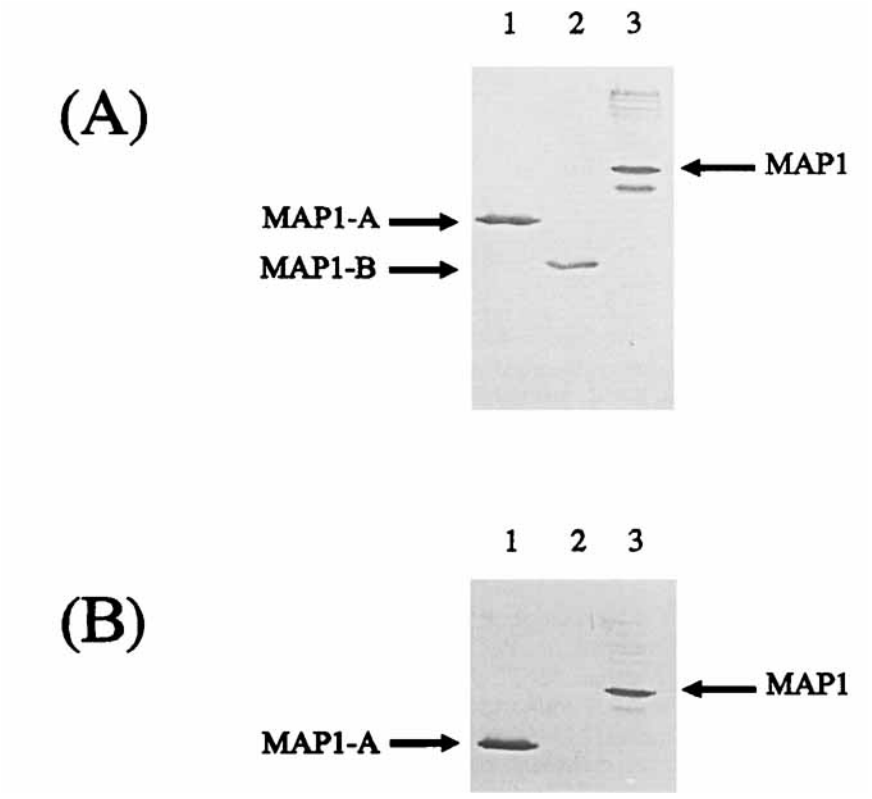

FIGURE 2. Reactivity of recombinant MAP1-A and MAP1-B on immunoblots with antisera to (A) *Cowdria ruminantium* (Senegal isolate) and (B) *Ehrlichia ovina*. Lane 1, MAP1-A; lane 2, MAP1-B; lane 3, *C. ruminantium* (Senegal isolate) antigens from endothelial cell cultures.

2 shows immunoblots of recombinant MAP1-A and MAP1-B reacted with antibodies to *C. ruminantium* and *E. ovina*. The MAP1-A region (which includes MAP1-B) contains one or more conserved epitopes which are recognized by antisera to *C. ruminantium, E. ovina, E. bovis, E. canis, E. chaffeensis,* and three MAP1-specific monoclonal antibodies (data not shown).

The MAP1-B region contains one or more epitopes with a high specificity for *C. ruminantium* antibodies. Only antibodies to *C. ruminantium, E. canis,* and *E. chaffeensis* reacted with MAP1-B. However, antisera to all *Ehrlichia* species that infect ruminants (*E. ovina, E. bovis,* and *E. phagocytophila*) did not react with MAP1-B.[28] The usefulness of a MAP1-B-based ELISA was evaluated with sera from sheep goats, and cattle experimentally infected with 9 different isolates of *C. ruminantium.* Reactivity of antisera with MAP1-B was expressed as a percentage of the reactivity of a serum sample of a sheep infected with *C. ruminantium* (C++). The cutoff value was fixed at twice the mean percentage reactivity of serum samples from noninfected control animals.[29,30] The mean percentage reactivity of negative sheep sera was 14.5

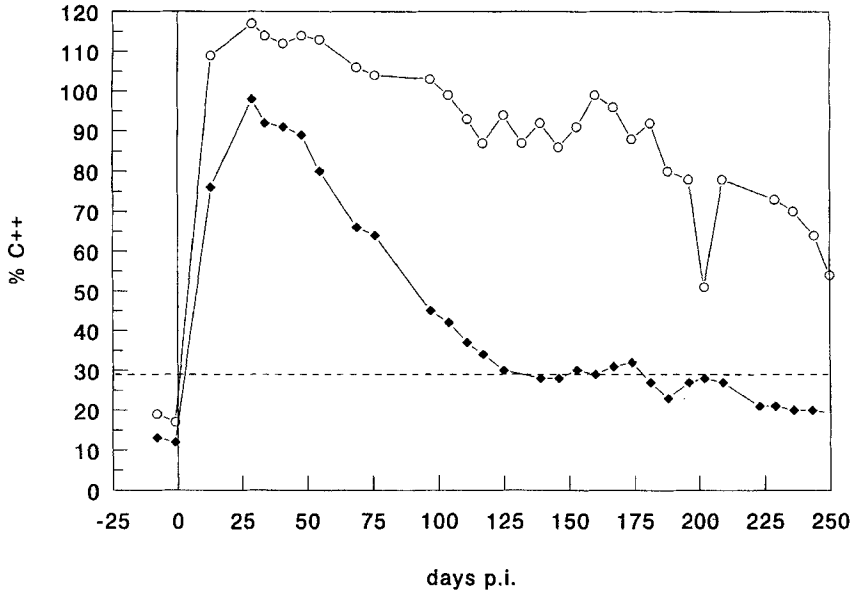

FIGURE 3. Reactivity of sequential serum samples obtained from two sheep experimentally infected with *C. ruminantium* (Senegal isolate). Reactivity with MAP1-B is expressed as percentage reactivity when compared to a positive control (C++). The dotted line represents the cutoff value. Circles: samples from sheep 110; diamonds: samples from sheep 144.

± 4.5 (n = 6). The cutoff value was therefore fixed at 29% for sheep sera. FIGURE 3 shows two representative examples of the reactivity of sheep antisera with MAP1-B, as a function of time postinfection (pi). To assess the sensitivity of the MAP1-B ELISA, serum samples of 64 experimental ruminants were taken 4 to 8 weeks after infection with *C. ruminantium*, and their reactivity with MAP1-B was determined. All serum samples were positive (data not shown).

Reactivity of Zimbabwean Sheep Sera with MAP1-B

It has been previously shown that sheep sera from areas in Zimbabwe that were considered to be free from *Amblyomma* ticks reacted with the MAP1 protein on immunoblots.[13] These reactions were considered to be false positives: 111 of these false-positive sheep sera, together with 55 negative sera and 10 sera from sheep experimentally infected with *C. ruminantium*, were retested using the MAP1-B antigen. The results obtained with these sheep sera are shown in FIGURE 4. False positivity was dramatically reduced, as only 9 out of 111 immunoblot-positive sera were detected by MAP1-B ELISA. Reactivity of the remaining false-positive sera ranged from 29%-43%. None of the 55 negative control sera exceeded the cutoff value, whereas all 10 positive controls reacted.

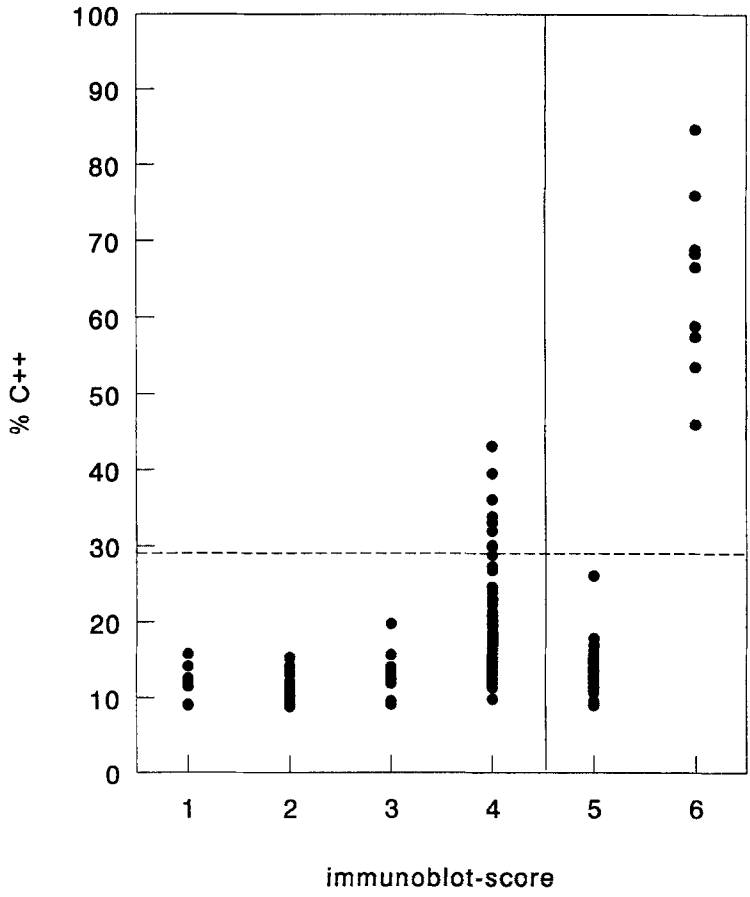

FIGURE 4. Reactivity of Zimbabwean sheep sera with MAP1-B in ELISA. Sera are grouped according to their immunoblot score.[13] Negative and positive controls are included in lanes 5 and 6, respectively. The dotted line represents the cutoff value. Lane 1, very weak positive sera (n = 8); lane 2, weak positive sera (n= 26); lane 3, positive sera (n = 15); lane 4, strongly positive sera (n = 62); lane 5, negative sera (n = 55); lane 6, positive controls (n = 10): sera from sheep experimentally infected with *C. ruminantium* (Welgevonden isolate).

Reactivity of Caribbean Sheep Sera with MAP1-B

Using competitive ELISA[18] and indirect ELISA,[20] sera from islands with no record of *C. ruminantium* infection and with low *A. variegatum* infestation[18,20] were positive. A panel of sheep sera previously tested with an indirect ELISA[20] were reacted with MAP1-B. FIGURE 5 shows the reactivity of these sera with MAP1-B. Sera are grouped per island. A high number of positive sera was found only with sheep from Antigua, where *C. ruminantium* has been isolated previously.[2] Using sera from islands where

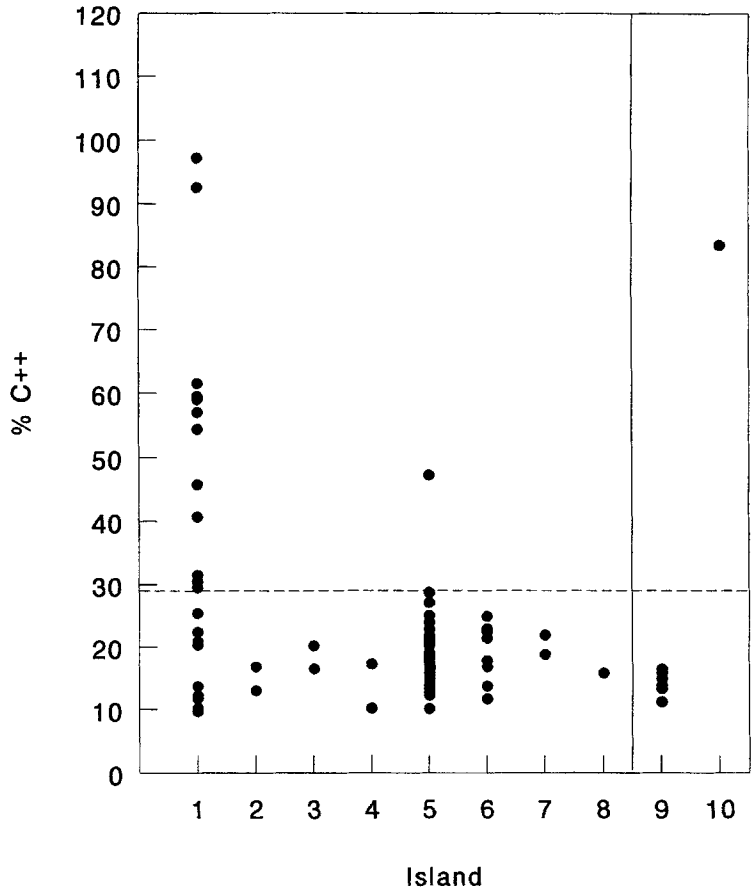

FIGURE 5. Reactivity of sheep sera from the Caribbean islands with MAP1-B in ELISA. Sera are grouped per island. Controls are included in lanes 9 and 10. The broken line represents the cutoff value. Lane 1, Antigua (n = 23); lane 2, Barbados (n = 3); lane 3, Dominica (n = 2); lane 4, Grenada (n = 2); lane 5, Martinique (n = 39); lane 6, Montserrat (n = 9); lane 7, St. Kitts (n = 2); lane 8, St. Vincent (n = 1); lane 9, negative control sera (n = 7); lane 10, positive control serum from a sheep experimentally infected with *C. ruminantium* (Gardel isolate).

C. ruminantium has not been isolated,[20] only one serum sample obtained from a sheep on the island of Martinique was positive.

DISCUSSION

An accurate assessment of the current distribution of *C. ruminantium* in Africa and in the Caribbean region is hampered by false-positive reactions in serological

tests, probably due to cross-reactive antibodies to *Ehrlichia* species. Positive sera were found in areas or on islands with no record of *Amblyomma* tick infestation. In order to obtain a serological assay with an improved specificity, the MAP1 protein of *C. ruminantium* was analyzed for the presence of immunogenic regions. Two of these regions were identified, and designated MAP1-A and MAP1-B. MAP1-A contained one or more epitopes responsible for cross-reactivity between MAP1 and antisera to *Ehrlichia* species, whereas MAP1-B contained one or more *C. ruminantium*-specific epitopes. Antibodies cross-reacting with MAP1-B were found in antisera to *E. chaffeensis* and *E. canis*. No cross-reactive antibodies were found in antisera to other *Ehrlichia* species. The observed pattern of cross-reactivity correlates with relationships based on rRNA sequence level, indicating that *C. ruminantium*, *E. canis*, and *E. chaffeensis* form a cluster separate from all other *Ehrlichia* species.[31] As *E. canis* or *E. chaffeensis* have never been isolated from ruminants, the cross-reactivity between these two *Ehrlichia* species and *C. ruminantium* MAP1-B is not expected to hamper heartwater serology.

Recombinant MAP1-B antigen is recognized by antibodies to 9 isolates of *C. ruminantium* originating from different geographic regions.[28] In addition, in a MAP1-B-based ELISA, as in other tests, antibodies to *C. ruminantium* were detected in sera from experimentally infected cattle, sheep, and goats at 12-24 days pi, and these sera remained positive for 50-250 days pi.[28] The MAP1-B-based ELISA had an excellent sensitivity, as sera from 64 ruminants experimentally infected with *C. ruminantium* all reacted with MAP1-B. The improved specificity of a MAP1-B based serological test was demonstrated using sera from areas or islands considered to be heartwater free, which were positive in other serological tests. False positivity of Zimbabwean sera was reduced to 9 out of 111 sera, and false positivity of Caribbean sera was reduced to 1 out of 58. The reason for these remaining false positives is not known. However, the reactivity with MAP1-B of most of these sera only slightly exceeded the cutoff value, indicating the possibility that a more accurate determination of the cutoff value after testing of more serum samples may lead to a further elimination of false-positive reactions. Finally, isolation and characterization of the organism that causes these putative false-positive reactions will be required.

SUMMARY

The major antigenic protein (MAP1) of *Cowdria ruminantium* was screened for immunogenic regions by expression of overlapping recombinant DNA clones of the gene encoding the MAP1 protein. Two regions, designated MAP1-A and MAP1-B, were recognized by all antisera to 9 different isolates of *C. ruminantium*. MAP1-A contained one or more epitopes responsible for false-positive reactions with *Ehrlichia* antisera in several serological tests for cowdriosis. Cross-reactivity with MAP1-B was limited to antisera to *Ehrlichia chaffeensis* and *Ehrlichia canis*. Antisera to *Ehrlichia* species that infect ruminants (*E. bovis*, *E. ovina*, and *E. phagocytophila*) did not recognize MAP1-B. The sensitivity of an indirect ELISA based on MAP1-B was found to be excellent, since all sera from animals experimentally infected with *C. ruminantium* (64 out of 64) reacted with MAP1-B. Validation of this ELISA was carried out with field sera obtained from sheep raised in heartwater-free areas

in Zimbabwe and from several Caribbean islands. Only 9 out of 111 samples from Zimbabwe, and 1 out of 58 samples from the Caribbean islands, which were considered to be false positives by immunoblot or indirect ELISA, reacted with MAP1-B. Thus, the ELISA based on MAP1-B is at present the most specific and sensitive serological test for cowdriosis.

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